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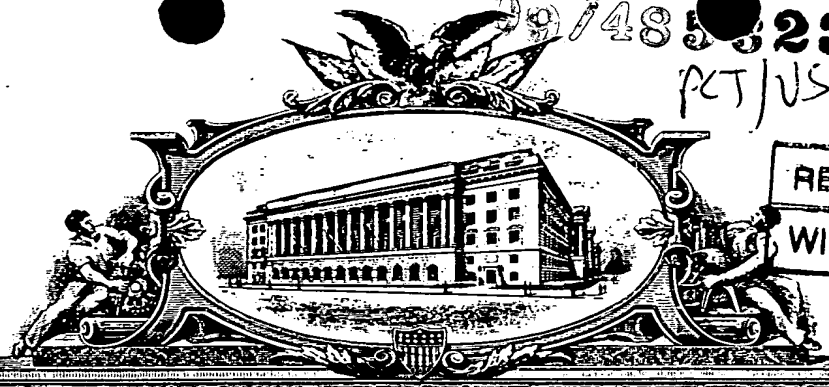

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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 C.F.R. 1.53(b)(2).

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TITLE OF THE INVENTION *(maximum 280 characters)*

EC-3, AN INHIBITOR OF $\alpha 4\beta 1$ AND $\alpha 4\beta 7$ INTEGRINS

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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_____.

Respectfully submitted,

Date: 8-15-97

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☐ Additional inventors are being named on separately numbered sheets attached hereto.

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EC-3, AN INHIBITOR OF $\alpha 4\beta 1$ AND $\alpha 4\beta 7$ INTEGRINS

Reference to Government Grant

The invention described herein was made, in part, in the course of work supported by the National Institutes of Health under Grant Nos. HL 45486 and HL 19055. The government has certain rights in the invention.

Field of the Invention

This invention generally relates to methods and compositions for modulating cell adhesion and for inhibiting the interaction between integrins and their ligands. In particular, the invention relates to peptides that specifically inhibit $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins.

Background of the Invention

A. Integrins

Integrins are a family of cell surface proteins that mediate adhesion between cells (cell-cell adhesion) and between cells and extracellular matrix proteins (cell-ECM adhesion). Integrins are heterodimeric structures composed of noncovalently bound α and β subunits. In humans there are at least 15 different α and eight different β subunits, and these can combine to form proteins with diverse biological activities and ligand specifities.

The integrins play important roles in many diverse biological processes including platelet aggregation, immune reactions, tissue repair, and tumor invasion. The integrins are, therefore, important targets for therapeutic intervention in human disease.

One example of the therapeutic targeting of integrins is the use of integrin inhibitors as antithrombotic agents.

Platelet aggregation is mediated by integrins on the platelet membrane surface GPIIb/IIIa complex (αIIb/β3). When platelets are activated GPIIb/IIIa binds fibrinogen, and this can lead to platelet aggregation and thrombus formation. Peptides and peptidomimetics that block the adhesion of GPIIb/IIIa to fibrinogen can prolong bleeding times and prevent thrombotic occlusion *in vivo*. One group of naturally occurring peptides which inhibit platelet aggregation by interfering with fibrinogen binding to GPIIb/IIIa has been called the "disintegrins."

B. Disintegrins

The disintegrins are a family of low molecular weight (5-9 kD) cysteine-rich, RGD (or KGD) containing peptides which have been isolated from the venom of various snakes (reviewed in Niewiarowski *et al.*, *Seminars in Hematology* **31(4)**:289-300 (1994)). RGD is a recognition site for many integrins, and the disintegrins inhibit fibrinogen binding to GPIIb/IIIa, as well as the binding of other ligands to RGD-dependant integrins on the surface of cells. Peptides modeled on the structure of disintegrins have potential clinical applications in the prevention and treatment of coronary thrombosis, stroke, and other vascular diseases.

A 49 amino acid disintegrin, called echistatin, has been isolated from the venom of *Echis carinatus* (Gan *et al.*, *J. Biol. Chem.* **263**:19827-32 (1988)). Like other disintegrins, echistatin contains an RGD sequence and inhibits GPIIb/IIIa binding of fibrinogen.

Disintegrin-like domains have been identified in mammalian proteins (Blobel and White, *Current Opinion Cell Biol.* **4**:760-65 (1992); Perry *et al.*, *Biochem J.* **286**:671-75 (1992)).

C. The α4 Integrins

The α4 integrins, $\alpha4\beta1$ and $\alpha4\beta7$, are expressed on leukocytes and lymphoid cells, and play a major role in inflammation and auto-immune diseases.

The $\alpha4\beta1$ integrin (which has also been called VLA-4, very late activation antigen 4) mediates cell adhesion to vascular cell adhesion molecule-1 (VCAM-1), an adhesive molecule belonging to the IgG superfamily which is expressed on endothelial cells. $\alpha4\beta1$ also binds to alternatively spliced variants of fibronectin containing connecting segment 1 (CS-1).

The $\alpha4\beta7$ integrin binds to CS-1, to VCAM-1, and also to the gut homing receptor mucosa addressin cell adhesion molecule-1 (MAdCAM-1).

It has been reported that the α4 subunit itself is a ligand for $\alpha4\beta1$ and $\alpha4\beta7$ integrins (Altevost *et al.*, *J. Exp. Med.* **182**:345-55 (1995)), suggesting that α4 integrins may play a role in leukocyte communication during the immune response.

Cytokine activated leukocytes express $\alpha4\beta1$ and $\alpha4\beta7$ integrins. Interaction of these integrins with VCAM-1 or MadCAM-1 (which are also up-regulated by cytokines) on endothelium mediates capillary infiltration by leukocytes, which can lead to tissue and organ destruction. Selectins and β2 integrins also contribute to this process.

Activation and up-regulation of $\alpha4\beta1$ or $\alpha4\beta7$ on lymphocytes or macrophages plays a significant role in the progression of following diseases: insulin dependent diabetes mellitus, multiple sclerosis, rheumatoid arthritis, ulcerative colitis, arteriosclerosis, asthma, allergy, and restenosis of arteries after surgery or angioplasty. The α4 integrins are therefore good targets for therapeutic intervention in a variety of inflammatory and auto-immune diseases.

D. The α4 Integrins as Therapeutic Targets

There are several animal models of inflammatory and autoimmune diseases in which endothelial infiltration by lymphocytes and organ destruction are blocked by anti-α4 monoclonal antibodies. As an example, anti-

α4 antibody inhibits lymphocyte infiltration of Langerhans islets in NOD mice, thus preventing development of spontaneous insulin dependent diabetes (Yang *et al.*, *Proc. Natl. Acad. Sci. USA* **91**:12604-08 (1994)). Anti-α4 monoclonal antibodies have also shown *in vivo* efficacy in animal models of asthma (Abraham *et al.*, *J. Clin. Invest.* **93**:776 (1994)), multiple sclerosis (Yednock *et al.*, *Nature* **356**:63 (1992)), inflammatory bowel disease (Podolsky *et al.*, *J. Clin. Invest.* **92**:372 (1993)), contact hypersensitivity (Chisholm *et al.*, *Eur. J. Immunol.* **23**:682 (1993)), and cardiac allograft rejection (Isobe *et al.*, *J. Immunol.* **153**:5810 (1994)).

There have been several attempts to develop synthetic inhibitors of α4β7 and α4β1. These inhibitors include cyclic RGD peptides and short peptides based on the sequences of MadCAM-1, VCAM-1, and CS-1. These peptides are typically active *in vitro* at the micromolar level.

Molossi *et al.* (*J. Clin. Invest.* **95**:2601-10 (June 1995)) reported that blockade of α4β1 (VLA-4) integrin binding to fibronectin with CS-1 peptide reduces accelerated coronary arteriopathy in rabbit cardiac allografts. The sequence of the CS-1 peptide was phenylacetic acid-Leu-Asp-Phe-d-Pro-amide.

Kogan *et al.* (WO 96/00581) reported that cyclic peptides modeled after a portion of the CS-1 peptide inhibited the binding of α4β1 integrin to VCAM-1 at concentrations of peptide less than about 10 μM.

Kogan *et al.* (U.S. Patent No. 5,510,332) reported that a peptide comprising the LDV domain of CS-1 peptide inhibited the binding of α4β1 integrin to VCAM-1 with an IC_{50} of 30 μM.

Shroff *et al.* (*Bioorganic and Medicinal Chemistry Letters* **6**(21):2495-2500 (1996)) reported a series of peptides based on the N-terminal domain of MAdCAM-1 inhibited the binding of HUT78 cells activated with Mn^{++} to MAdCAM-1 with an IC_{50} of 5 to >1000 μM. Cyclic peptides based on an N-terminal conserved motif in VCAM-1 have also been reported to inhibit

VCAM/$\alpha 4\beta 1$ mediated leukocyte adhesion (Wang *et al.*, *Proc. Natl. Acad. Sci. USA* **92**:5714 (1995)).

Vanderslice *et al.* (*J. Immunol.* **158**:1710-18 (1997)) recently reported that a cyclic binding of lymphocytes to VCAM and CS-1 with an IC_{50} of 1-3 μM.

There is a need for potent and specific inhibitors of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins, and for methods of specifically inhibiting the binding of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ to specific cellular ligands.

Summary of the Invention

The present invention relates to the discovery of the EC-3 protein, an extremely potent antagonist of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins, which inhibits adhesion of cells expressing these integrins to natural ligands VCAM-1 and MAdCAM-1 with IC_{50} of 3-30 nM.

The invention provides a substantially purified protein named EC-3, isolated from *E. carinatus* venom and characterized by:

(a) an apparent molecular weight of about 14,667 Da, as determined by mass spectrometry;

(b) elution from a C-18 HPLC column at about 40% acetonitrile; and

(c) the ability to specifically inhibit $\alpha 4\beta 1$ integrin binding to VCAM-1.

The invention also provides a substantially purified EC-3A peptide, isolated from EC-3 protein and characterized by:

(a) a molecular mass of about 4066 Da in its ethyl pyridylethylated form, as determined by mass spectrometry; and

(b) elution from a C-18 HPLC column at about 43% acetonitrile, in its ethylpyridylethylated form.

The invention further provides a substantially purified EC-3B peptide, isolated from EC-3 protein and characterized by:

(a) a molecular mass of about 8521-8614 Da in its ethyl pyridylethylated form, as determined by mass spectrometry;

(b) elution from a C-18 HPLC column at about 46% acetonitrile, in its ethylpyridylethylated form; and

(c) the ability to specifically inhibit $\alpha 4\beta 1$ integrin binding to VCAM-1.

One embodiment of the invention is a substantially purified peptide comprising the sequence SEQ ID NO:1, or a biologically active fragment or derivative thereof.

Another embodiment of the invention is a substantially purified peptide comprising the sequence SEQ ID NO:2, or a biologically active fragment or derivative thereof. In some preferred embodiments the peptide is from about 3 to about 20 amino acids. In some preferred embodiments the biologically active fragment has the sequence X-Y-M-L-D-Z, where X is H or a blocking group, Y is zero or more amino acids, and Z is OH or zero or more amino acids. In a most preferred embodiment the peptide has the sequence SEQ ID NO:7. In another most preferred embodiment the peptide has the sequence SEQ ID NO:8.

A further embodiment of the invention is a substantially purified protein from *E. carinatus* comprising two subunits, wherein one subunit has the sequence SEQ ID NO:1 and one subunit has the sequence SEQ ID NO:2, or a biologically active fragment or derivative thereof.

Another aspect of the invention is a substantially purified nucleic acid encoding a protein or peptide according to the invention. One embodiment of the invention is a vector comprising a nucleic acid encoding a protein or peptide according to the invention. Another embodiment of the invention is a recombinant cell comprising a nucleic acid encoding a protein or peptide according to the invention.

The invention further provides an antibody which specifically binds to a protein or peptide according to the invention. The antibody may be a monoclonal antibody or a polyclonal antibody or an antibody fragment that is

capable of binding antigen. One aspect of the invention is a hybridoma that produces a monoclonal antibody which specifically binds to a protein or peptide according to the invention.

Another aspect of the invention is a substantially purified echistatin polypeptide in which the Arg-Gly-Asp residues at positions 24-26 are replaced by Met-Lev-Asp, or a biologically active fragment or derivative thereof.

The invention further provides a method of isolating a peptide that binds to an integrin of interest from venom comprising:

(a) dissolving venom in a solvent,

(b) centrifuging the dissolved venom to remove high molecular weight proteins,

(c) fractionating the supernatant from step (b),

(d) immobilizing the fractions from step (c) on a solid support,

(e) adding detectably labeled cells which express the integrin of interest to the immobilized fractions,

(f) detecting the number of cells bound to each immobilized fraction, and

(g) isolating peptide from those fractions which showed enhanced cell binding in step (f).

Another aspect of the invention is a composition comprising a pharmaceutically acceptable carrier and a protein or peptide according to the invention.

The invention encompasses a method of inhibiting the binding of $\alpha 4\beta 1$ integrin to VCAM-1 comprising contacting a cell that expresses $\alpha 4\beta 1$ with an effective amount of a protein or peptide according to the invention.

The invention also encompasses a method of inhibiting the binding of $\alpha 4\beta 7$ integrin to VCAM-1 comprising contacting a cell that expresses $\alpha 4\beta 7$ with an effective amount of a protein or peptide according to the invention.

The invention further encompasses a method of inhibiting the binding of $\alpha 4\beta 7$ integrin to MadCAM-1 comprising contacting a cell that expresses $\alpha 4\beta 7$ with an effective amount of a protein or peptide according to one of claims 1-9.

A preferred embodiment of the invention is a method of inhibiting the interaction between cells expressing $\alpha 4\beta 1$ integrin and VCAM-1 in a patient in need of such treatment comprising administration of a therapeutically effective amount of a composition comprising a pharmaceutically acceptable carrier and a protein or peptide according to the invention.

Another preferred embodiment of the invention is a method of inhibiting the interaction between cells expressing $\alpha 4\beta 7$ integrin and VCAM-1 or MadCAM-1 in a patient in need of such treatment comprising administration of a therapeutically effective amount of a composition comprising a pharmaceutically acceptable carrier and a protein or peptide according to the invention.

The invention also constitutes the use of a protein or peptide according to the invention for the preparation of a medicament for inhibiting the interaction between cells expressing $\alpha 4\beta 1$ integrin and VCAM-1 or for inhibiting the interaction between cells expressing $\alpha 4\beta 7$ integrin and VCAM-1 or MadCAM-1.

Other aspects and advantages of the present invention are described in the drawings and in the following detailed description of the preferred embodiments thereof.

Description of the Drawings

Figure 1 shows the HPLC fractionation of *E. carinatus* venom on a C-18 HPLC column eluted with an acetonitrile gradient. Fractions containing the disintegrin echistatin, the EC-3 protein, and the echicetin protein (which binds to GPIb/IX receptors on platelets) are indicated by arrows.

Figure 2 shows the elution profile of reduced and alkylated EC-3 from a reverse phase HPLC column. Figure 2A shows the elution profile of

reduced and alkylated EC-3 applied to the C-18 column and eluted with an acetonitrile gradient. Figure 2B shows the profile of a control solution applied to the same column.

Figure 3 shows a comparison of the amino acid sequence of EC-3B (SEQ ID NO:2), with two short disintegrins eristostatin (SEQ ID NO:3) and echistatin (SEQ ID NO:4), and two long disintegrins flavoridin (SEQ ID NO:5) and kistrin (SEQ ID NO:6). Boxes show the positions of conserved cysteine residues. The active site of the disintegrins (RGD) as well as the corresponding position (MLD) in EC-3B are underlined.

Figure 4 shows the effect of EC-3 on Jurkat cell adhesion to immobilized VCAM-1 in the presence (filled circles) or absence (open circles) of 1 mM Mn^{++}.

Figure 5 shows the effect of EC-3 on CHO (α4+, α5-) cell adhesion to immobilized VCAM-1.

Figure 6 shows the effect of GRGDSP (SEQ ID NO:9) and GRGESP (SEQ ID NO:10) peptides and anti-α4 monoclonal antibody HP 2/4 on Jurkat cell adhesion to immobilized EC-3.

Figure 7 shows the activity of ethylpyridylated EC-3A (filled circles) and EC-3B (open circles) in the inhibition of Jurkat cell adhesion to immobilized VCAM-1.

Detailed Description of the Invention

The present invention relates to the discovery of a novel protein, called EC-3, which is a potent inhibitor of $\alpha4\beta1$ and $\alpha4\beta7$ integrins.

EC-3 protein was purified from *Echis carinatus* viper venom. EC-3 is an extremely potent antagonist of $\alpha4\beta1$ and $\alpha4\beta7$ integrins and it inhibits adhesion of cells expressing these integrins to natural ligands VCAM-1 and MadCAM-1 with IC_{50} = 3-30 nM. Its blocking activity resembles that of some monoclonal antibodies but is 2 to 3 orders of magnitude higher than most synthetic antagonists. The EC-3 inhibitory effect is not RGD dependent but is

competed by HP 2/4 and HP 2/1 monoclonal antibodies recognizing epitopes on $\alpha 4$.

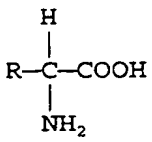
EC-3 is composed of at least two types of subunits, EC-3A (Mr = 3751.2 Da) and EC-3B (Mr = 7468 Da), linked by intramolecular S-S bridge(s). Activity resides in subunit B, which shows some homology with disintegrins, especially with respect to conserved cysteines. The conserved RGD sequence of integrins is replaced, however, by MLD in EC-3B. EC-3A shows some homology, including the consensus Zn binding site, with Zn-metalloproteinase precursors (such as hemorrhagic toxins, fibrolase, and adamalysin), suggesting that it may have metalloproteinase activity.

EC-3 appears to be a member of the family of proteins called ADAMS (a disintegrin and metalloproteinase family), proteins which are widely distributed and play roles in fertilization and development (Wolfsberg and White, *Developmental Biology* **180**:349-401 (1996)).

A. Definitions

The following definitions, of terms used throughout the specification, are intended as an aid to understanding the scope and practice of the present invention.

A "**peptide**" is a compound comprised of amino acid residues covalently linked by peptide bonds. Amino acids have the following general structure:



Amino acids are classified into seven groups on the basis of the side chain R: (1) aliphatic side chains, (2) side chains containing a hydroxylic (OH) group, (3) side chains containing sulfur atoms, (4) side chains containing an acidic or amide group, (5) side chains containing a basic group, (6) side chains containing an aromatic ring, and (7) proline, an imino acid in which the side chain is fused

to the amino group. Peptides comprising a large number of amino acids are sometimes called "**polypeptides**". The EC-3A and EC-3B subunits of EC-3 are peptides.

A "**protein**" is a polypeptide which plays a structural or functional role in a biological system. Proteins comprise one or more peptides. EC-3 is a protein.

"**Fibrinogen**" is a blood plasma glycoprotein, which is involved in platelet aggregation and fibrin formation.

"**Integrins**" are a family of heterodimeric cell surface proteins which mediate adhesion between cells and between cells and extracellular matrix proteins.

"**Disintegrins**" are a family of RGD containing peptides isolated from snake venoms which inhibit the binding of various ligands to RGD dependent integrins.

"**Echistatin**" is a 49 amino acid disintegrin isolated from *Echis carinatus* venom.

"**VCAM-1**" is an abbreviation for vascular cell adhesion molecule-1, a member of the IgG protein superfamily which is a cellular ligand for the $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins.

"**MAdCAM-1**" is an abbreviation for mucosa addressin cell adhesion molecule-1, a member of the IgG protein superfamily having a mucin component. MadCAM-1 is a cellular ligand for the $\alpha 4\beta 7$ integrin.

"**EC-3 protein**" is a heterodimeric protein comprising an EC-3A subunit and an EC-3B subunit. The term "**EC-3 peptide**" as used herein encompasses the EC-3 protein as well as the EC-3A and EC-3B subunits.

"**Homology**" means similarity of sequence reflecting a common evolutionary origin. Peptides or proteins are said to have homology, or similarity, if a substantial number of their amino acids are either (1) identical, or (2) have a chemically similar R side chain. Nucleic acids are said to have homology if a substantial number of their nucleotides are identical.

"**Substantial amino acid sequence homology**" means an amino acid sequence homology greater than about 30 percent, preferably greater than about 60%, more preferably greater than about 80%, and most preferably greater than about 90 percent.

"**Substantially purified peptide**" or "**substantially purified protein**" means a peptide or protein which is substantially free of those compounds that are normally associated therewith in its natural state (*e.g.*, other proteins or peptides, nucleic acids, carbohydrates, lipids). "Substantially purified" is not meant to exclude artificial or synthetic mixtures with other compounds, or the presence of impurities which do not interfere with biological activity, and which may be present, for example, due to incomplete purification, addition of stabilizers, or compounding into a pharmaceutically acceptable preparation.

A "**biologically active fragment**" of an EC-3 peptide is a fragment derived from an EC-3 peptide which retains at least one biological activity of the EC-3 peptide.

A "**biologically active derivative**" of an EC-3 peptide is any analogue, variant, derivative, or mutant which is derived from an EC-3 peptide, which has substantial amino acid sequence homology with the EC-3 peptide, and which retains at least one biological property of the EC-3 peptide. Different variants of the EC-3 peptide may exist in nature. These variants may be allelic variations characterized by differences in the nucleotide sequences of the structural gene coding for EC-3 peptides, or may involve differential splicing or post-translational modification. The skilled artisan can produce derivatives having single or multiple amino acid substitutions, deletions, additions, or replacements. These derivatives may include, *inter alia*: (a) derivatives in which one or more amino acid residues are substituted with conservative or non-conservative amino acids, (b) derivatives in which one or more amino acids are added to an EC-3 peptide, (c) derivatives in which one or more of the amino acids includes a substituent group, and (d) derivatives in which the EC-3 peptide is fused with another peptide such as serum albumin. The techniques for

obtaining these derivatives, including genetic (suppressions, deletions, mutations, etc.), chemical, and enzymatic techniques, are known to persons having ordinary skill in the art.

Biologically active fragments and biologically active derivatives of EC-3 peptides are intended to be included within the scope of this invention.

A "**blocking group**" is any group capable of blocking the N-terminal amino group of a peptide. A preferred blocking group is an alkyl group; a most preferred blocking group is an acetyl group.

"**HPLC**" is an acronym for high performance liquid chromatography.

"**IC_{50}**" is the concentration of a biologically active agent such as a peptide, which inhibits 50% of the activity obtained in the absence of the agent.

"**RGD**" is the one letter designation for the amino acid sequence Arg-Gly-Asp. RGD is a recognition site for many disintegrins. A related recognition site is "**KGD**": Lys-Gly-Asp.

"**MLD**" is the one letter designation of the amino acid sequence Met-Leu-Asp, which occurs in the EC-3 protein.

A "**nucleic acid**" is a polymeric compound comprised of covalently linked subunits called nucleotides. Nucleic acid includes polyribonucleic acid (RNA) and polydeoxyribonucleic acid (DNA), both of which may be single-stranded or double-stranded. DNA includes cDNA, genomic DNA, synthetic DNA, and semi-synthetic DNA. The sequence of nucleotides that encodes a protein is called the sense sequence.

"**Isolated nucleic acid**" means a nucleic acid which is substantially free of those compounds that are normally associated therewith in its natural state. "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds, or the presence of impurities which do not interfere with biological activity, and which may be present, for example, due to incomplete purification, addition of stabilizers, or compounding into a pharmaceutically acceptable preparation.

The phrase "**a nucleic acid which hybridizes at high stringency**" means that the hybridized nucleic acids are able to withstand a washing under high stringency conditions. An example of high stringency washing conditions for DNA-DNA hybrids is 0.1X SSC, 0.5% SDS at 68°C. Other conditions of high stringency washing are known to persons having ordinary skill in the art.

"**Regulatory region**" means a nucleic acid sequence which regulates the expression of a nucleic acid. A regulatory region may include sequences which are naturally responsible for expressing a particular nucleic acid (a homologous region) or may include sequences of a different origin (responsible for expressing different proteins or even synthetic proteins). In particular, the sequences can be sequences of eukaryotic or viral genes or derived sequences which stimulate or repress transcription of a gene in a specific or non-specific manner and in an inducible or non-inducible manner. Regulatory regions include origins of replication, RNA splice sites, enhancers, transcriptional termination sequences, signal sequences which direct the polypeptide into the secretory pathways of the target cell, and promoters.

A regulatory region from a "**heterologous source**" is a regulatory region which is not naturally associated with the expressed nucleic acid. Included among the heterologous regulatory regions are regulatory regions from a different species, regulatory regions form a different gene, hybrid regulatory sequences, and regulatory sequences which do not occur in nature, but which are designed by one having ordinary skill in the art.

"**Operatively linked to a regulatory region**" means that the peptide or polypeptide coding region is connected to transcriptional and translational regulatory sequences in such a way as to permit polypeptide expression when the appropriate molecules (such as activator proteins and polymerases) are present in a cell or cell free system.

A "**vector**" is any means for the transfer of a nucleic acid according to the invention into a host cell. The term "vector" includes both viral and nonviral means for introducing the nucleic acid into a prokaryotic or

eukaryotic cell *in vitro*, *ex vivo* or *in vivo*. Non-viral vectors include plasmids, liposomes, electrically charged lipids (cytofectins), DNA-protein complexes, and biopolymers. Viral vectors include retrovirus, adeno-associated virus, pox, baculovirus, vaccinia, herpes simplex, Epstein-Barr and adenovirus vectors. In addition to a nucleic acid according to the invention, a vector may also contain one or more regulatory regions, and/or selectable markers useful in selecting, measuring, and monitoring nucleic acid transfer results (transfer to which tissues, duration of expression, etc.).

A "**recombinant cell**" is a cell which contains a nucleic acid which is not naturally present in the cell. "Recombinant cell" includes higher eukaryotic cells such as mammalian cells, lower eukaryotic cells such as yeast cells, prokaryotic cells, and archaebacterial cells.

"**Antibody**" as used herein includes monoclonal and polyclonal antibodies as well as fragments capable of binding antigen, including but not limited to Fab and $F(ab)_2$ fragments.

"**Pharmaceutically acceptable carrier**" includes diluents and fillers which are pharmaceutically acceptable for method of administration, are sterile, and may be aqueous or oleaginous suspensions formulated using suitable dispersing or wetting agents and suspending agents. The particular pharmaceutically acceptable carrier and the ratio of active compound to carrier are determined by the solubility and chemical properties of the composition, the particular mode of administration, and standard pharmaceutical practice.

B. Proteins and Peptides

The present invention provides substantially purified EC-3 peptides, including the EC-3 protein, the EC-3A and EC-3B peptides, and biologically active fragments and derivatives of EC-3, EC-3A, and EC-3B. The invention also provides methods of isolation and peptides isolated from other venoms having biological activity similar to EC-3.

The peptides of the present invention may be recombinant peptides, natural peptides, or synthetic peptides. Each peptide is characterized

by a reproducible single molecular weight and/or multiple set of molecular weights, chromatographic response and elution profile, amino acid composition and sequence, and biological activity.

The peptides of the present invention may be isolated from natural sources, such as viper venom, using the methods disclosed herein.

The peptides of the invention may also be chemically synthesized, using, for example, solid phase synthesis methods.

In conventional solution phase peptide synthesis, the peptide chain can be prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence. The use of various N-protecting groups, *e.g.*, the carbobenzyloxy group of the t-butyloxycarbonyl group (B)C), various coupling reagents (*e.g.*, dicyclohexylcarbodiimide or carbonyldimidazole, various active esters, *e.g.*, esters of N-hydroxyphthalimide or N-hydroxy-succinimide, and the various cleavage reagents, *e.g.*, trifluoroacetic acid (TFA), HCl in dioxane, boron tris-(trifluoracetate) and cyanogen bromide, and reaction in solution with isolation and purification of intermediates is well-known classical peptide methodology. The preferred peptide synthesis method follows conventional Merrifield solid-phase procedures. See Merrifield, *J. Amer. Chem. Soc.* **85**:2149-54 (1963) and *Science* **50**:178-85 (1965). Additional information about the solid phase synthesis procedure can be had by reference to the treatise by Steward and Young ("Solid Phase Peptide Synthesis," W.H. Freeman & Co., San Francisco, 1969, and the review chapter by Merrifield in *Advances in Enzymology* **32**:221-296, F.F. Nold, Ed., Interscience Publishers, New York, 1969; and Erickson and Merifield, *The Proteins* 2:255 *et seq.* (ed. Neurath and Hill), Academic Press, New York, 1976. The synthesis of peptides by solution methods is described in Neurath *et al.*, eds. (*The Proteins*, Vol. II, 3d Ed., Academic Press, NY (1976)).

Crude peptides may be purified using preparative HPLC. The amino terminus may be blocked according, for example, to the methods described by Yang *et al.* (*FEBS Lett.* **272**:61-64 (1990)).

Peptide synthesis includes both manual and automated techniques employing commercially available peptide synthesizers. Fragments and derivatives of EC-3 peptides may be prepared by chemical synthesis and biological activity can be tested using the methods disclosed herein.

Alternatively, the peptides of the invention may be prepared utilizing recombinant DNA technology, which comprises combining a nucleic acid encoding the peptide thereof in a suitable vector, inserting the resulting vector into a suitable host cell, recovering the peptide produced by the resulting host cell, and purifying the polypeptide recovered.

In some embodiments, the peptides of the present invention may be used in the form of a pharmaceutically acceptable salt.

Suitable acids which are capable of forming salts with the peptides include inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acid and the like; and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid, naphthalene sulfonic acid, sulfanilic acid and the like.

Suitable bases capable of forming salts with the peptides include inorganic bases such as sodium hydroxide, ammonium hydroxide, potassium hydroxide and the like; and organic bases such as mono-, di- and tri-alkyl and aryl amines (*e.g.*, triethylamine, diisopropyl amine, methyl amine, dimethyl amine and the like) and optionally substituted ethanol-amines (*e.g.*, ethanolamine, diethanolamine and the like).

C. Nucleic Acids

The present invention provides substantially purified nucleic acids which encode peptides according to the invention.

The techniques of recombinant DNA technology are known to those of ordinary skill in the art. General methods for the cloning and expression of recombinant molecules are described in Maniatis (*Molecular*

Cloning, Cold Spring Harbor Laboratories, 1982), and in Ausubel (*Current Protocols in Molecular Biology*, Wiley and Sons, 1987), which are incorporated by reference.

Having the EC-3A and EC-3B amino acid sequences disclosed herein, one of skill in the art can prepare a synthetic gene that encodes the peptide of interest. Synthetic genes may be synthesized directly on a DNA synthesizer, or may be synthesized as complementary oligonucleotides which are ligated together to form the synthetic gene. Alternatively, the native gene encoding the peptides of the invention may be isolated from genomic or cDNA libraries. As an example, based upon the amino acid sequences disclosed herein, one of skill in the art can prepare suitable oligonucleotide probes and polymerase chain reaction (PCR) primers, which can be used to screen a cDNA library prepared from *E. carinatus* venom glands. Positive clones are purified and sequenced to confirm their identity.

The nucleic acids encoding EC-3 peptides may be operatively linked to one or more regulatory regions. Regulatory regions include promoters, polyadenylation signals, translation initiation signals (Kozak regions), termination codons, peptide cleavage sites, and enhancers. The regulatory sequences used must be functional within the cells of the vertebrate to be immunized. Selection of the appropriate regulatory region or regions is a routine matter, within the level of ordinary skill in the art.

Promoters that may be used in the present invention include both constitutive promoters and regulated (inducible) promoters. The promoters may be prokaryotic or eukaryotic depending on the host. Among the prokaryotic (including bacteriophage) promoters useful for practice of this invention are lacI, lacZ, T3, T7, lambda P_r, P_l, and trp promoters. Among the eukaryotic (including viral) promoters useful for practice of this invention are ubiquitous promoters (*e.g.* HPRT, vimentin, actin, tubulin), intermediate filament promoters (*e.g.* desmin, neurofilaments, keratin, GFAP), therapeutic gene promoters (*e.g.* MDR type, CFTR, factor VIII), tissue-specific promoters (*e.g.* actin promoter in smooth muscle cells), promoters which respond to a stimulus

(*e.g.* steroid hormone receptor, retinoic acid receptor), tetracycline-regulated transcriptional modulators, cytomegalovirus immediate-early, retroviral LTR, metallothionein, SV-40, E1a, and MLP promoters. Tetracycline-regulated transcriptional modulators and CMV promoters are described in WO 96/01313, US 5,168,062 and 5,385,839, the contents of which are incorporated herein by reference.

Examples of polyadenylation signals that can be used in the present invention include but are not limited to SV40 polyadenylation signals and LTR polyadenylation signals.

Fragments and derivatives of EC-3 peptides may be prepared using recombinant DNA technology. The biological activity of the fragments and derivatives can be assayed using the methods disclosed herein.

D. Antibodies

The present invention provides antibodies against EC-3 peptides. These antibodies may be monoclonal antibodies or polyclonal antibodies. The present invention includes chimeric, single chain, and humanized antibodies, as well as Fab fragments and the products of an Fab expression library.

Polyclonal antibodies may be generated against the intact protein or peptide, or against a fragment, derivative, or epitope of the protein or peptide. Antibodies may be obtained following the administration of the protein, polypeptide, fragment, derivative, or epitope to an animal, using the techniques and procedures known in the art.

Monoclonal antibodies may be prepared using the method of Mishell, B.B., *et al.*, *Selected Methods In Cellular Immunology*, (W.H. Freeman, ed.) San Francisco (1980). Briefly, a peptide of the present invention is used to immunize spleen cells of Balb/C mice. The immunized spleen cells are fused with myeloma cells. Fused cells containing spleen and myeloma cell characteristics are isolated by growth in HAT medium, a medium which kills both parental cells, but allows the fused products to survive and grow.

Antibodies may be used to purify the peptides according to the invention, using immunoaffinity techniques which are well known by those of skill in the art.

E. Isolation of Related Peptides from Venoms

The present invention provides a method for isolating peptides that bind to an integrin of interest from the venom of snakes or other organisms. This method comprises the steps of:

(a) dissolving venom in a solvent,

(b) centrifuging the dissolved venom to remove high molecular weight proteins,

(c) fractionating the supernatant from step (b),

(d) immobilizing the fractions from step (c) on a solid support,

(e) adding detectably labeled cells which express the integrin of interest to the immobilized fractions,

(f) detecting the number of cells bound to each immobilized fraction, and

(g) isolating peptide from those fractions which showed enhanced cell binding in step (f).

This method may be used to isolate peptides from the venom of species including but not limited to vipers and other snakes (including *Agkistrodon acutus, Agkistrodon halys blomhoffi, Agkistrodon contortrix mokasen, Bitis arietans, Bitis caudalis, Bitis gabonica, Bitis g. rhinoceros, Bothrops asper, Bothrops alternata, Bothrops atrox, Bothrops cotiara, Bothrops jararaca, Bothrops newiedi, Bothrops medusa, Bothrops schlegli, Cerastes cerastes, Cerastes vipera, Crotalus adamanteus, C. atrox, C. basilicus, C. durissus totonatacus, C. h. horridus, C. m. molossus, C. ruber, C. scutalatus, C. v. cereberus, C. v. helleri, C. v. lutosus, C. v. oreganus, Echis carinatus sochurecki, Eristicophis macmahoni, Pseudocerastes persicus, Sistrurus m. barbouri, Sistrurus c. terqeminus, Trimeresurus flavoviridis, Trimeresurus*

gramineus, Vipera lebetina, Vipera ammondytes, Vipera palastinae, and *Vipera r. russelli*, leeches, ticks, and from other organisms.

F. Methods of Treatment

The present invention provides methods of inhibiting the binding of $\alpha4\beta1$ or $\alpha4\beta7$ integrins to a ligand. A preferred embodiment is a method of inhibiting the interaction between cells expressing $\alpha4\beta1$ and/or $\alpha4\beta7$ integrins and a ligand, in a patient in need of such treatment, comprising administration of a therapeutically effective amount of a composition comprising one or more peptides according to the invention or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers include physiologically tolerable or acceptable diluents, excipients, solvents, adjuvants, or vehicles, for parenteral injection, for intranasal or sublingual delivery, for oral administration, for recal or topical administration or the like. The compositions are preferably sterile and nonpyrogenic. Examples of suitable carriers include but are not limited to water, saline, dextrose, mannitol, lactose, or other sugars, lecithin, albumin, sodium glutamate cystein hydrochloride, ethanol, polyols (propyleneglycol, polyethyleneglycol, glycerol, and the like), vegetable oils (such as olive oil), injectable organic esters such as ethyl oleate, ethoxylated isosteraryl alcohols, polyoxyehtylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum methahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances, and the like.

The pharmaceutical compositions may also contain minor amounts of nontoxic auxiliary substances such as wetting agents, emulsifying agents, pH buffering agents, antibacterial and antifungal agents (such as parabens, chlorobutanol, phenol, sorbic acid, and the like). If desired, absorption enhancing or delaying agents (such as liposomes, aluminum monostearate, or gelatin) may be used. The compositions can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions.

The compositions may be administered by any convenient route which will result in delivery to the blood stream in an amount effective for inhibiting $\alpha 4\beta 4$ and $\alpha 4\beta 7$ mediated adhesion, including orally, rectally, parenterally (intravenously, intramuscularly, intraarterially, or subcutaneously), intracisternally, intravaginally, intraperitoneally, locally (powders, ointments or drops), or as a buccal or nasal spray or aerosol. The compositions can also be delivered through a catheter for local delivery at a target site, or via a biodegradable polymer. The compositions may also be complexed to ligands, or antibodies, for targeted delivery of the compositions.

The compositions are most effectively administered parenterally, preferably intravenously. For intravenous administration, they may be dissolved in any appropriate intravenous delivery vehicle containing physiologically compatible substances, such as sodium chloride, glycine, and the like, having a buffered pH compatible with physiologic conditions. Such intravenous delivery vehicles are known to those skilled in the art. In a preferred embodiment, the vehicle is a sterile saline solution. If the peptides are sufficiently small (*e.g.*, less than about 8-10 amino acids) other preferred routes of administration are intranasal, sublingual, and the like.

The compositions according to the invention can be administered in any circumstance in which inhibition of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrin function is desirable. Disease states which may be treated include but are not limited to diabetes, multiple sclerosis, rheumatoid arthritis, ulcerative colitis, arteriosclerosis, asthma, allergy, and restenosis of the arteries following surgery or angioplasty. Because the $\alpha 4$ integrins are expressed on various cancer cells, including leukemia, melanomas, lymphomas, and sarcomas, inhibitors of $\alpha 4$ binding may also be useful in the treatment of some forms of cancer.

The amount of peptide administered depends upon the degree of integrin inhibition that is desired. Those skilled in the art will derive appropriate dosages and schedules of administration to suit the specific circumstances and needs of the patient. Typically, dosages are between about 0.001 mg/kg and about 100 mg/kg body weight. In some embodiments dosages

are between about 0.01 mg/kg and about 10 mg/kg body weight. In some embodiments dosages are between about 0.05 mg/kg and about 5 mg/kg body weight.

Examples

The following examples illustrate the invention. These examples are illustrative only, and do not limit the scope of the invention.

EXAMPLE 1

Purification of EC-3

Lyophilized *Echis carinatus* venom (25 mg, obtained from Latoxan Serpentarium, Rosans 05150 France) was dissolved in 1 ml 0.1% trifluoroacetic acid (TFA). The solution was centrifuged for 5 minutes at 5000 rpm to remove the insoluble high molecular weight proteins. The pellet was discarded, and the supernatant was applied to a C-18 HPLC column. The column was eluted with an acetonitrile gradient (0-80%)

Each eluted fraction was lyophilized, then dissolved in water. The protein concentration in each fraction was estimated using the BCA assay (Pierce). Two μg of protein from each fraction was immobilized (in separate wells) on a microtiter plate overnight at 4°C in PBS (phosphate buffered saline).

Jurkat cells were labeled by incubation with 12.5 μM CMFDA (5-chloromethylfluorescein diacetate) in HBSS (Hanks' balanced salt solution) buffer containing 1% BSA (bovine serum albumin) for 15 minutes at 37°C. Cells were washed three times to remove excess CMFDA.

The labeled Jurkat cells were added to the microtiter plate (1 x 10^5 cells per well), and the plate was incubated for 30 minutes at 37°C. After incubation, unbound cells were removed by aspiration and the wells were washed three times with HBSS/BSA buffer.

Bound cells were lysed by the addition of 0.5% Triton X-100, and fluorescence was measured in a Cytofluor 2350 fluorescence plate reader

(Millipore) using a 485 nm EX (excitation) filter and a 530 nm EM (emission) filter.

Figure 1 shows the HPLC fractionation of venom components. Echistatin was identified as a strong antagonist of the fibrinogen receptor, showing high inhibitory activity (IC_{50} = 130 nM) in an ADP-induced platelet aggregation assay.

The venom fraction eluting at approx. 40% was designated EC-3. EC-3 was further purified by an additional step of reverse phase HPLC using a C-18 column and an acetonitrile gradient (0-60% over 45 minutes time). The EC-3 protein is a cysteine rich protein with a molecular weight of about 14,667 Da.

EXAMPLE 2

Characterization of EC-3

A. Subunit Structure and Molecular Mass

EC-3 was reduced and pyridylethylated by adding to 100 μg protein, 6 M guanidine hydrochloride, 4 mM EDTA, 0.1M Tris-HCl, pH 8.5, 3.2 mM dithiothreitol, and 2 μl of vinyl pyridine. The reaction mixture was incubated for two hours at room temperature in the dark, then applied to a C-18 HPLC column and eluted with an acetonitrile gradient.

As shown in Figure 2, the EC-3 eluted as two peptides. These peptides were designated EC-3A (eluting at 43% acetonitrile) and EC-3B (eluting at 46% acetonitrile).

The molecular mass of EC-3A and EC-3B, as determined by mass spectrometry and substraction of the vinyl pyridine Mr, was 3751 and 7468 respectively.

B. Amino Acid Sequence

The complete amino acid sequence of EC-3A was determined by Edman degradation of pyridylethylated EC-3A, using an Applied Biosystems Procise instrument.

The EC-3A subunit is 37 amino acids, has a calculated molecular weight of 3751 Da, and has the following sequence:

Val Ala Ser Thr Met Ala His Glu Met Gly His Asn Leu Gly Met Asp His Asp Gly Ser Gln Cys Asn Cys Gly Gly Ala Gly Cys Val Met Ser Ala Thr Ile Thr Arg (SEQ ID NO:1).

The N-terminal sequence analysis of ethylpyridylethylated EC-3B yielded the sequence:

Asn Ser Val His Pro Cys Cys Asp Pro Val Lys Cys Glu Pro Arg Glu Gly Glu His Cys Ile Ser Gly Pro Cys Cys Arg Asn Cys Lys Phe Leu Asn Ala Gly Thr Ile Cys Lys Arg Ala Met Leu Asp Gly (SEQ ID NO:11)

Ethylpyridylethylated EC-3B was degraded with CNBr under the following reaction conditions: 10 mg/ml protein in 70% formic acid, 100 mg/ml CNBr, for six hours, under N_2 atmosphere, in the dark. The resulting peptides were separated by reverse phase HPLC.

Fragment CNBr-2 displayed two sequences, which were interpreted as:

Asn Ser Val His Pro Cys Cys Asp Pro Val Lys Cys Glu Pro Arg Glu Gly Glu His Cys Ile Ser Gly Pro Cys Cys (SEQ ID NO:12); and

Leu Asp Gly Leu Asn Asp Tyr Cys Thr Gly Lys Ser Ser Asp Cys Pro Arg Asn Pro Asn Tyr Arg (Gly Gly Phe Lys) (SEQ ID NO:13).

There was some uncertainty regarding the assignment of the last four residues of SEQ ID NO:13. Combining the overlapping sequences yielded the following amino acid sequence, which has the expected molecular mass (68 amino acids, with a calculated molecular weight of 7468 Da) for EC-3B:

Asn Ser Val His Pro Cys Cys Asp Pro Val Lys Cys Glu Pro Arg Glu Gly Glu His Cys Ile Ser Gly Pro Cys Cys Arg Asn Cys Lys Phe Leu Asn Ala Gly Thr Ile Cys Lys Arg Ala Met Leu Asp Gly

Leu Asn Asp Tyr Cys Thr Gly Lys Ser Ser Asp Cys Pro Arg Asn Pro Asn Tyr Arg Gly Gly Phe Lys (SEQ ID NO:2).

The EC-3A subunit does not show homology with the disintegrins but shows some homology, including the consensus Zn binding site, with Zn-metalloproteinase precursors.

The EC-3B subunit has some sequence homology, most notably in the number and arrangement of cysteines, with several disintegrins, as shown in Figure 3. An interesting structural feature emerges when the EC-3B subunit is aligned (via the cysteine residues) with the disintegrins: the active site "RGD" of the disintegrins is occupied by the sequence MLD in EC-3.

EXAMPLE 3

Biological Activity of EC-3

A. Effect of EC-3 on Jurkat Cell Adhesion to Immobilized VCAM-1

The following experiment demonstrated that EC-3 inhibits the adhesion of Jurkat cells to VCAM-1.

Recombinant human VCAM-1 (0.5 μg/well) was immobilized in the wells of an ELISA plate overnight in PBS buffer. The plate was blocked using HBSS buffer containing 1% BSA. CMFDA-labelled Jurkat cells (1 x 10^5 cells per sample) were added to the wells in the presence or absence of EC-3 in HBSS buffer containing 1% BSA. The plate was incubated for 30 minutes at 37°C. Unbound cells were removed by aspiration and the wells were washed with HBSS buffer. The bound cells were lysed with 0.5% Triton X-100, and fluorescence was measured. Percent inhibition was calculated by comparing the fluorescence obtained for adhered cells in the absence (0% inhibition) and presence of EC-3. Figure 4 shows the percent inhibition with increasing concentrations of EC-3, in the presence (filled circles) or absence (open circles) of 1 mM Mn^{++}.

B. Effect of EC-3 on α4B2 Cell Adhesion to Immobilized VCAM-1.

The following experiment demonstrated that EC-3 inhibits the adhesion of Chinese hamster ovary cells, which are α5 deficient (B2 cells) and have been transfected with human α4 integrin, to VCAM-1.

Chinese hamster ovary cells transfected with human α4 integrin were provided by Dr. Y. Takada (Scripps Research Institute, La Jolla, CA). Recombinant human VCAM-1 (0.5 μg/well) was immobilized in the wells of an ELISA plate overnight in PBS buffer. The plate was blocked using HBSS buffer containing 1% BSA. CMFDA-labelled α4B2 cells (1 x 10^5 cells per sample) were added to the wells in the presence or absence of EC-3 in HBSS buffer containing 1% BSA. The plate was incubated for 1 hour at 37°C. Unbound cells were removed by aspiration and the wells were washed with HBSS buffer. The bound cells were lysed with 0.5% Triton X-100, and fluorescence was measured. Figure 5 shows the percent inhibition (determined as in Example 3A, above) with increasing concentrations of EC-3.

C. Effect of GRGDSP (SEQ ID NO:9) and GRGESP (SEQ ID NO:10) Peptides and HP 2/4 Monoclonal Antibody on Jurkat Cell Adhesion to Immobilized EC-3.

The HP 2/4 monoclonal antibody, which blocks α4, was provided by Dr. F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). EC-3 (1 μg/well) was immobilized overnight in PBS buffer in ELISA plates, and the plates were blocked with HBSS buffer containing 1% BSA. CMFDA-labelled Jurkat cells (1 x 10^5/well) were added to the wells in the presence of 1 mM peptides or 1 μg/sample HP 2/4 antibody in HBSS/BSA buffer. Plates were incubated 30 minutes at 37°C. Unbound cells were removed by aspiration and the wells were washed with HBSS buffer. Bound cells were lysed by 0.5% Triton X-100, and fluorescence was measured.

As shown in Figure 6, the adhesion of Jurkat cells to immobilized EC-3 was inhibited by the HP 2/4 monoclonal antibody. These results suggest

that EC-3 and HP 2/4 may recognize the same epitope on $\alpha 4$ integrin. Adhesion was not inhibited by GRGDSP (or the control peptide GRGESP). This is in agreement with the absence of an RGD sequence in EC-3.

D. Comparison of the Anti-adhesive Effects of EC-3 and Echistatin

The following experiment demonstrated the comparative inhibitory effects of EC-3 and echistatin (a disintegrin isolated from *E. carinatus* venom) on (1) cell adhesion to various ligands, and (2) ADP induced platelet aggregation.

The effect of each peptide on cell adhesion was determined as in Examples 3A and 3B. Briefly, ligands were immobilized or ELISA plates then incubated with cells labeled with CMFDA in the presence of various concentrations of EC-3 or echistatin. Cells that attached to the plates were lysed with detergent, and fluorescence intensity was measured. Percent inhibition was measured at each concentration and IC_{50} was determined. Three cell lines expressing the $\alpha 4\beta 1$ receptor were tested: Jurkat, Ramos and Chinese hamster ovary cells (CHO cells) transfected with a human $\alpha 4$ gene (which express $\beta 1$ integrin and are $\alpha 5$ deficient).

The results are shown in Table 1. In all experiments EC-3 blocked adhesion of the $\alpha 4\beta 1$ cell lines to immobilized VCAM-1 with IC_{50} 20 - 90 nM. Echistatin was not active in this system. Echistatin is a potent inhibitor of $\alpha 5\beta 1$ (adhesion of K562 cells to fibronectin, IC_{50} = 20 nM), $\alpha v\beta 3$ and $\alpha IIb\beta 3$ integrin interaction with fibrinogen and vitronectin (IC_{50} = 50 nM). EC-3 showed a low antagonistic activity to $\alpha 5\beta 1$ and $\alpha IIb\beta 3$ but had no effect on $\alpha v\beta 3$.

Table 1

Cells (receptor(s))	Immobilized ligand	IC_{50} EC-3	IC_{50} Echistatin
Jurkat ($\alpha4\beta1$, $\alpha5\beta1$)	VCAM-1	20 nM* 50 nM**	>10,000 nM
Jurkat	fibronectin	280 nM	> 1,000 nM
K562 ($\alpha5\beta1$)	fibronectin	300 nM	20 nM
Ramos ($\alpha4\beta1$)	VCAM-1	60 nM	> 1,000 nM
8866 ($\alpha4\beta7$)	MadCAM-1	70 nM	> 1,000 nM
CHO $\alpha4+\alpha5-$	VCAM-1	90 nM	>10,000 nM
CHO $\alpha v\beta3$	vitronectin	>1,000 nM	50 nM
CHO $\alpha IIb\beta3$	fibrinogen	500 nM	50 nM

* tested in the presence of 1 mM manganese
** tested in the absence of 1 mM manganese

E. Platelet Aggregation Assay

Blood from a healthy donor was collected into a syringe containing sodium citrate as an anticoagulant. The anticoagulated blood was centrifuged at 400g for 17 minutes, and platelet rich plasma (PRP) was removed by aspiration. The aggregation of platelets in PRP in the presence of 30 μM ADP was measured in the absence and presence of echistatin and EC-3, using an aggregation meter (Sinco).

Echistatin and EC-3 inhibited ADP induced platelet aggregation with an IC_{50} of 130 nM and 1,000 nM, respectively.

EXAMPLE 4

Biological Activity of EC-3 Derivatives

A. Biological Activity of the EC-3B Subunit

Ethylpyridylated EC-3A and EC-3B were eluted from an HPLC column and freeze-dried, then tested for the ability to inhibit Jurkat cell adhesion to immobilized VCAM-1, using the assay described in Example 3A.

As shown in Figure 7, EC-3A (filled circles) was not active in this assay. The EC-3B peptide (open circles), even though structurally disrupted, showed some activity but this activity was 100-fold lower than that of native EC-3.

B. Biological Activity of EC-3 Peptides

The following peptide was synthesized using a solid phase method:

Cys Lys Arg Ala Met Leu Asp Gly Leu Asn Asp Tyr Cys (SEQ ID NO:7)

This peptide was cyclic, a disulfide bond being formed between the terminal cysteines, and inhibited Jurkat cell adhesion to immobilized VCAM-1 with an IC_{50} of 300-500 μM.

Additional peptides, comprising fragments and derivatives of EC-3 such as Met Leu Asp Gly Leu (SEQ ID NO:8), are synthesized and inhibitory activity is measured using assays such as those described in Example 3.

C. Biological Activity of Modified Echistatin Peptides

Using standard methods of recombinant DNA technology, a modified echistatin polypeptide is produced. In the modified echistatin, the Arg-Gly-Asp residues at positions 24-26 are replaced by Met-Leu-Asp. The biological activity of the modified echistatin, as well as fragments and derivatives thereof, is determined.

All references discussed herein are incorporated by reference. One skilled in the art will readily appreciate that the present

invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: EC-3 AN INHIBITOR OF ALPHA 4 BETA 1 AND ALPHA 4 BETA 7 INTEGRINS

(iii) NUMBER OF SEQUENCES: 14

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Val Ala Ser Thr Met Ala His Glu Met Gly His Asn Leu Gly Met Asp
1               5                  10                  15

His Asp Gly Ser Gln Cys Asn Cys Gly Gly Ala Gly Cys Val Met Ser
            20                  25                  30

Ala Thr Ile Thr Arg
        35
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Asn Ser Val His Pro Cys Cys Asp Pro Val Lys Cys Glu Pro Arg Glu
1               5                   10                  15

Gly Glu His Cys Ile Ser Gly Pro Cys Cys Arg Asn Cys Lys Phe Leu
            20                  25                  30

Asn Ala Gly Thr Ile Cys Lys Arg Ala Met Leu Asp Gly Leu Asn Asp
        35                  40                  45

Tyr Cys Thr Gly Lys Ser Ser Asp Cys Pro Arg Asn Pro Asn Tyr Arg
    50                  55                  60

Gly Gly Phe Lys
65
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Gln Glu Glu Pro Cys Ala Thr Gly Pro Cys Cys Arg Arg Cys Lys Phe
1               5                   10                  15

Lys Arg Ala Gly Lys Val Cys Arg Val Ala Arg Gly Asp Trp Asn Asp
            20                  25                  30

Asp Tyr Cys Thr Gly Lys Ser Cys Asp Cys Pro Arg Asn Pro Trp Asn
        35                  40                  45

Gly
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Glu Cys Glu Ser Gly Pro Cys Cys Arg Asn Cys Lys Phe Leu Lys Glu
1               5                   10                  15

Gly Thr Ile Cys Lys Arg Ala Arg Gly Asp Asp Met Asp Asp Tyr Cys
            20                  25                  30

Asn Gly Lys Thr Cys Asp Cys Pro Arg Asn Pro His Lys Gly Pro Ala
        35                  40                  45

Thr
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Gly Glu Glu Cys Asp Cys Gly Ser Pro Ser Asn Pro Cys Cys Asp Ala
1               5                   10                  15

Ala Thr Cys Lys Leu Arg Pro Gly Ala Gln Cys Ala Asp Gly Leu Cys
            20                  25                  30

Cys Asp Gln Cys Arg Phe Lys Lys Lys Thr Gly Ile Cys Arg Ile Ala
        35                  40                  45

Arg Gly Asp Phe Pro Asp Asp Arg Cys Thr Gly Leu Ser Asn Asp Cys
    50                  55                  60

Pro Arg Trp Asn Asp Leu
65                  70
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Lys Glu Cys Asp Cys Ser Ser Pro Glu Asn Pro Cys Cys Asp Asp
1 5 10 15

Ala Thr Cys Lys Leu Arg Pro Gly Ala Gln Cys Gly Glu Gly Leu Cys
20 25 30

Cys Glu Gln Cys Lys Phe Ser Arg Ala Gly Lys Ile Cys Arg Ile Pro
35 40 45

Arg Gly Asp Met Pro Asp Asp Arg Cys Thr Gly Gln Ser Ala Asp Cys
50 55 60

Pro Arg Tyr His
65

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:
(A) NAME/KEY: Disulfide-bond
(B) LOCATION: 1..13

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Cys Lys Arg Ala Met Leu Asp Gly Leu Asn Asp Tyr Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Leu Asp Gly Leu
1   5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Arg Gly Asp Ser Pro
1   5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Arg Gly Glu Ser Pro
1   5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Asn Ser Val His Pro Cys Cys Asp Pro Val Lys Cys Glu Pro Arg Glu
1               5                  10                  15

Gly Glu His Cys Ile Ser Gly Pro Cys Cys Arg Asn Cys Lys Phe Leu
            20                  25                  30

Asn Ala Gly Thr Ile Cys Lys Arg Ala Met Leu Asp Gly
        35                  40                  45
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Asn Ser Val His Pro Cys Cys Asp Pro Val Lys Cys Glu Pro Arg Glu
1               5                  10                  15

Gly Glu His Cys Ile Ser Gly Pro Cys Cys
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Leu Asp Gly Leu Asn Asp Tyr Cys Thr Gly Lys Ser Ser Asp Cys Pro
1               5                  10                  15

Arg Asn Pro Asn Tyr Arg Gly Gly Phe Lys
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Asn Ser Val His Pro Cys Cys Asp Pro Val Lys Cys Glu Pro Arg Glu
1               5                   10                  15

Gly Glu His Cys Ile Ser Gly Pro Cys Cys Arg Asn Cys Lys Phe Leu
            20                  25                  30

Asn Ala Gly Thr Ile Cys Lys Arg Ala Met Leu Asp Gly Leu Asn Asp
        35                  40                  45

Tyr Cys Thr Gly Lys Ser Ser Asp Cys Pro Arg Asn Pro Asn Tyr Arg
    50                  55                  60
```

CLAIMS

WE CLAIM

1. A substantially purified EC-3 protein isolated from *E. carinatus* venom, characterized by:

(a) an apparent molecular weight of about 14,667 Da, as determined by mass spectrometry;

(b) elution from a C-18 HPLC column at about 40% acetonitrile; and

(c) the ability to specifically inhibit $\alpha 4\beta 1$ integrin binding to VCAM-1.

2. A substantially purified EC-3A peptide isolated from EC-3 protein, characterized by:

(a) a molecular mass of about 4066 Da in its ethyl pyridylethylated form, as determined by mass spectrometry; and

(b) elution from a C-18 HPLC column at about 43% acetonitrile, in its ethylpyridylethylated form.

3. A substantially purified EC-3B peptide isolated from EC-3 protein, characterized by:

(a) a molecular mass of about 8521-8614 Da in its ethyl pyridylethylated form, as determined by mass spectrometry;

(b) elution from a C-18 HPLC column at about 46% acetonitrile, in its ethylpyridylethylated form; and

(c) the ability to specifically inhibit $\alpha 4\beta 1$ integrin binding to VCAM-1.

4. A substantially purified peptide comprising the sequence SEQ ID NO:1, or a biologically active fragment or derivative thereof.

5. A substantially purified peptide comprising a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:14, or a biologically active fragment or derivative thereof.

6. A substantially purified protein from *E. carinatus* comprising two subunits, wherein one subunit has the sequence SEQ ID NO:1 and one subunit comprises the sequence SEQ ID NO:14, or a biologically active fragment or derivative thereof.

7. A biologically active fragment according to claim 5 having the sequence X-Y-M-L-D-Z, where X is H or a blocking group, Y is zero or more amino acids, and Z is OH or zero or more amino acids.

8. A biologically active fragment according to claim 5 wherein said fragment is a peptide having from about 3 to about 20 amino acids.

9. A fragment according to claim 8 having the sequence SEQ ID NO:7.

10. A fragment according to claim 8 having the sequence SEQ ID NO:8.

11. A substantially purified nucleic acid encoding the protein or peptide of one of claims 1-10.

12. A vector comprising the nucleic acid of claim 11.

13. A recombinant cell comprising the nucleic acid of claim 11.

14. An antibody which specifically binds to one of the proteins or peptides of claims 1-10.

15. The antibody of claim 14 wherein said antibody is a monoclonal antibody.

16. A hybridoma that produces the antibody of claim 15.

17. The antibody of claim 14 wherein said antibody is a polyclonal antibody.

18. A substantially purified eichistatin polypeptide in which the Arg-Gly-Asp residues at positions 24-26 are replaced by Met-Leu-Asp, or a biologically active fragment or derivative thereof.

19. A method of isolating a peptide that binds to an integrin of interest from venom comprising:

(a) dissolving venom in a solvent,

(b) centrifuging the dissolved venom to remove high molecular weight proteins,

(c) fractionating the supernatant from step (b),

(d) immobilizing the fractions from step (c) on a solid support,

(e) adding detectably labeled cells which express the integrin of interest to the immobilized fractions,

(f) detecting the number of cells bound to each immobilized fraction, and

(g) isolating peptide from those fractions which showed enhanced cell binding in step (f).

20. A composition comprising a pharmaceutically acceptable carrier and the protein or peptide of one of claims 1-10, or a pharmaceutically acceptable salt thereof.

21. A method of inhibiting the binding of $\alpha 4\beta 1$ integrin to VCAM-1 comprising contacting a cell that expresses $\alpha 4\beta 1$ with an effective amount of a protein or peptide according to one of claims 1-10, or a pharmaceutically acceptable salt thereof.

22. A method of inhibiting the binding of $\alpha 4\beta 7$ integrin to VCAM-1 comprising contacting a cell that expresses $\alpha 4\beta 7$ with an effective amount of a protein or peptide according to one of claims 1-10, or a pharmaceutically acceptable salt thereof.

23. A method of inhibiting the binding of $\alpha 4\beta 7$ integrin to MadCAM-1 comprising contacting a cell that expresses $\alpha 4\beta 7$ with an effective amount of a protein or peptide according to one of claims 1-10, or a pharmaceutically acceptable salt thereof.

24. A method of inhibiting the interaction between cells expressing $\alpha 4\beta 1$ integrin and VCAM-1 in a patient in need of such treatment comprising administration of a therapeutically effective amount of a composition according to claim 20.

25. A method of inhibiting the interaction between cells expressing $\alpha 4\beta 7$ integrin and VCAM-1 or MadCAM-1 in a patient in need of such treatment comprising administration of a therapeutically effective amount of a composition according to claim 20.

EC-3, AN INHIBITOR OF $\alpha4\beta1$ and $\alpha4\beta7$ INTEGRINS

ABSTRACT

The invention relates to the identification, purification, and characterization of a novel protein, EC-3, from *Echis carinatus* viper venom. EC-3 is an extremely potent antagonist of $\alpha4\beta1$ and $\alpha4\beta7$ integrins. The invention further relates to methods of using EC-3, or a biologically active fragment or derivative thereof, to inhibit the interaction between cells expressing the $\alpha4\beta1$ and/or $\alpha4\beta7$ integrins and cellular ligands.

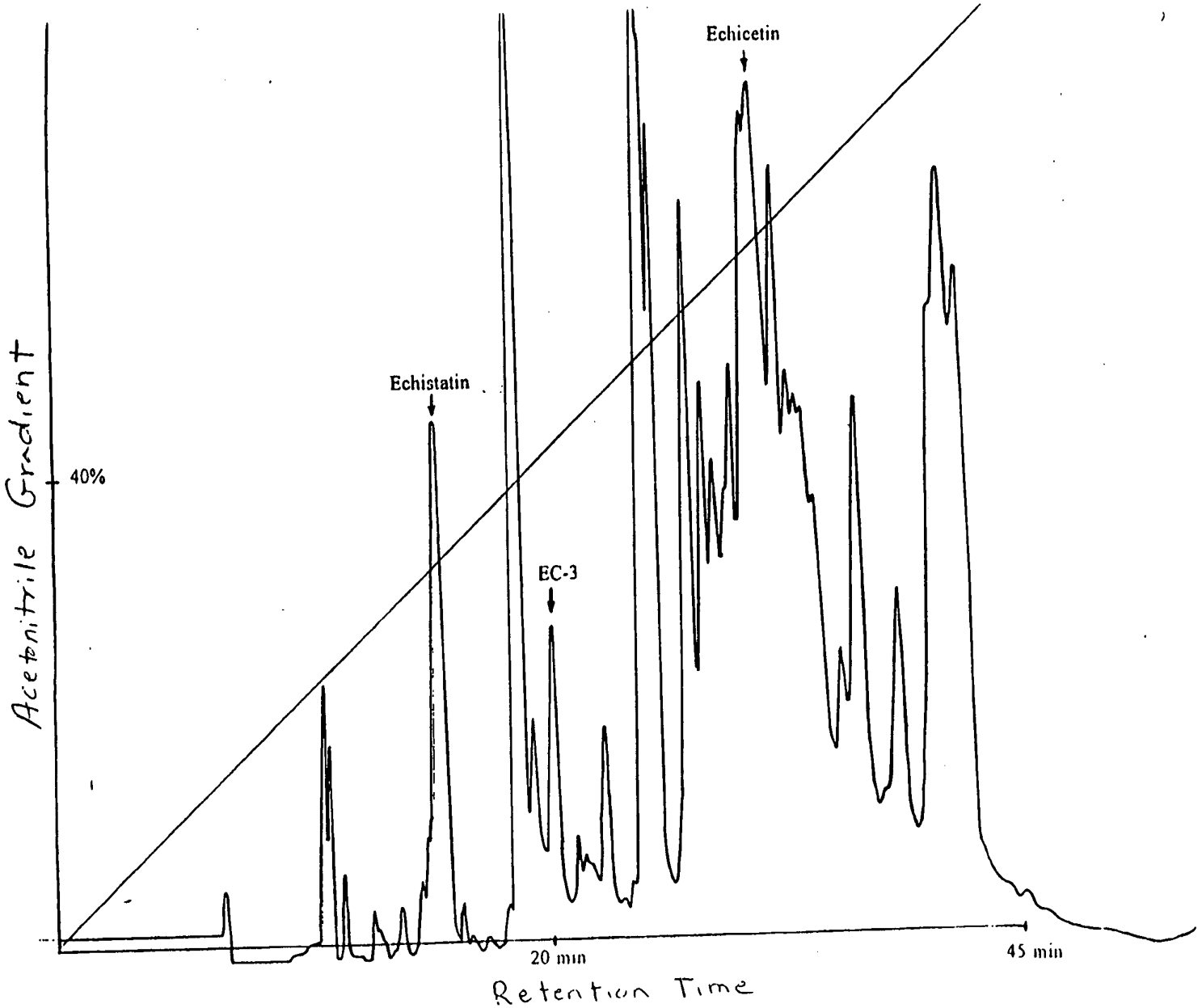

Fig. 1

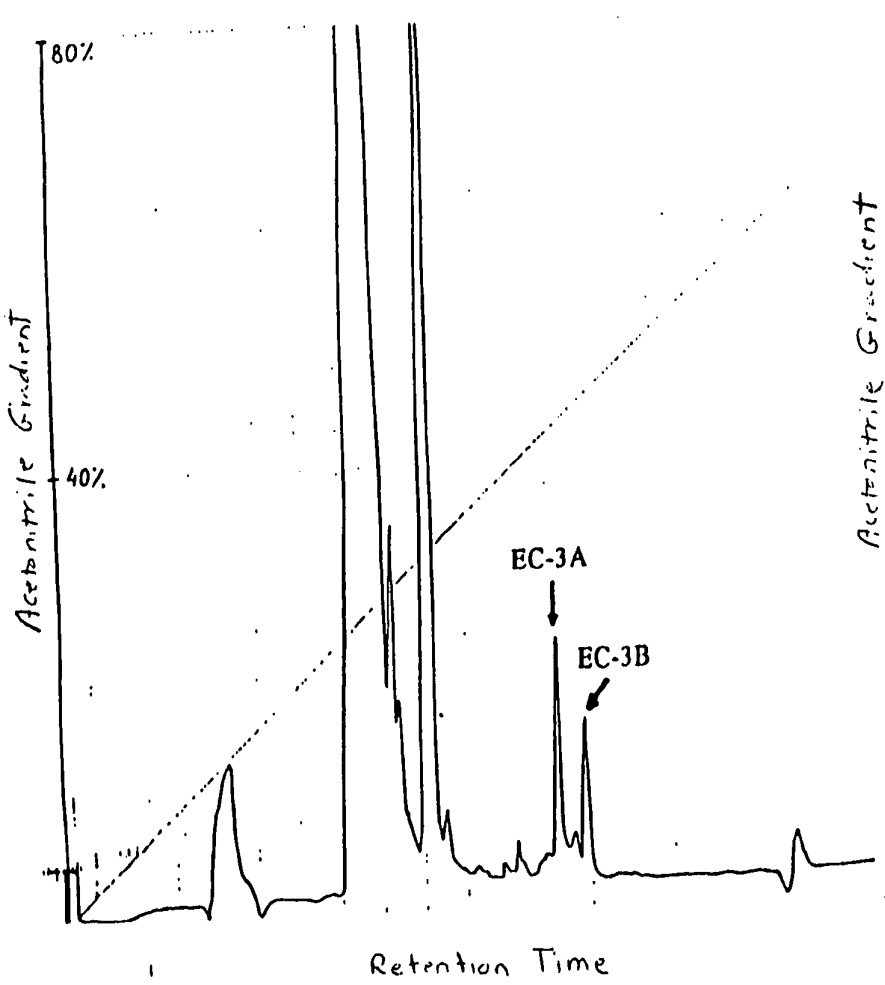

Fig. 2A

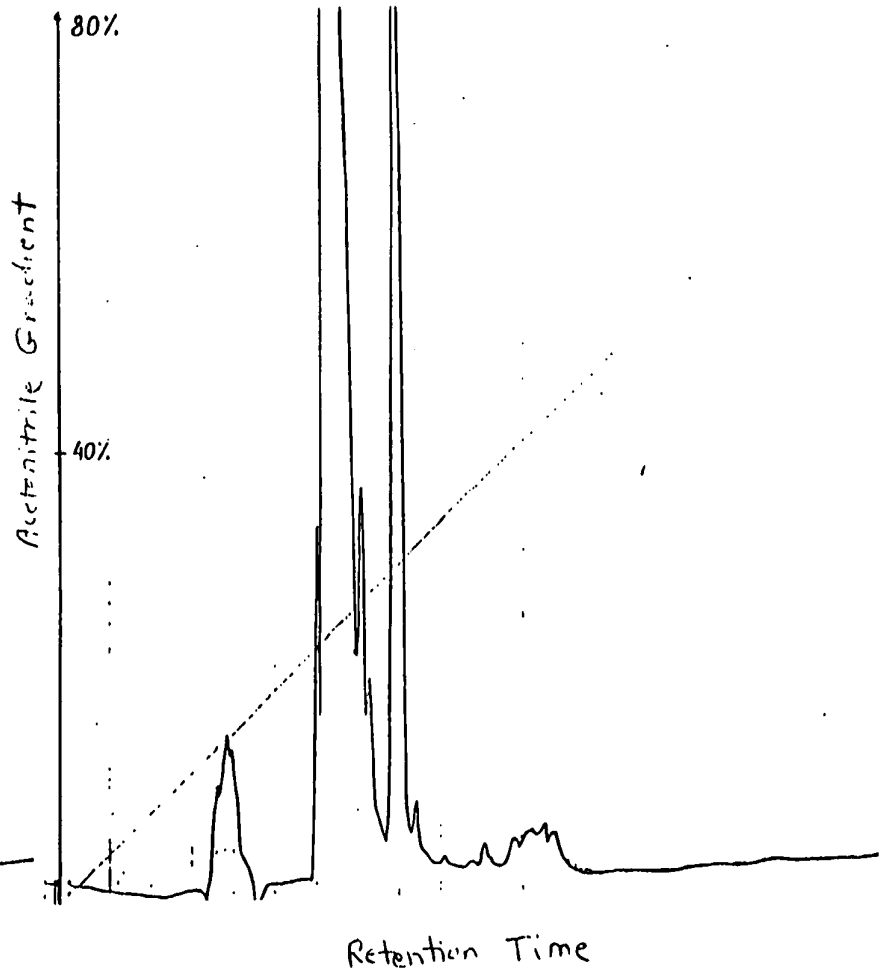

Fig. 2B

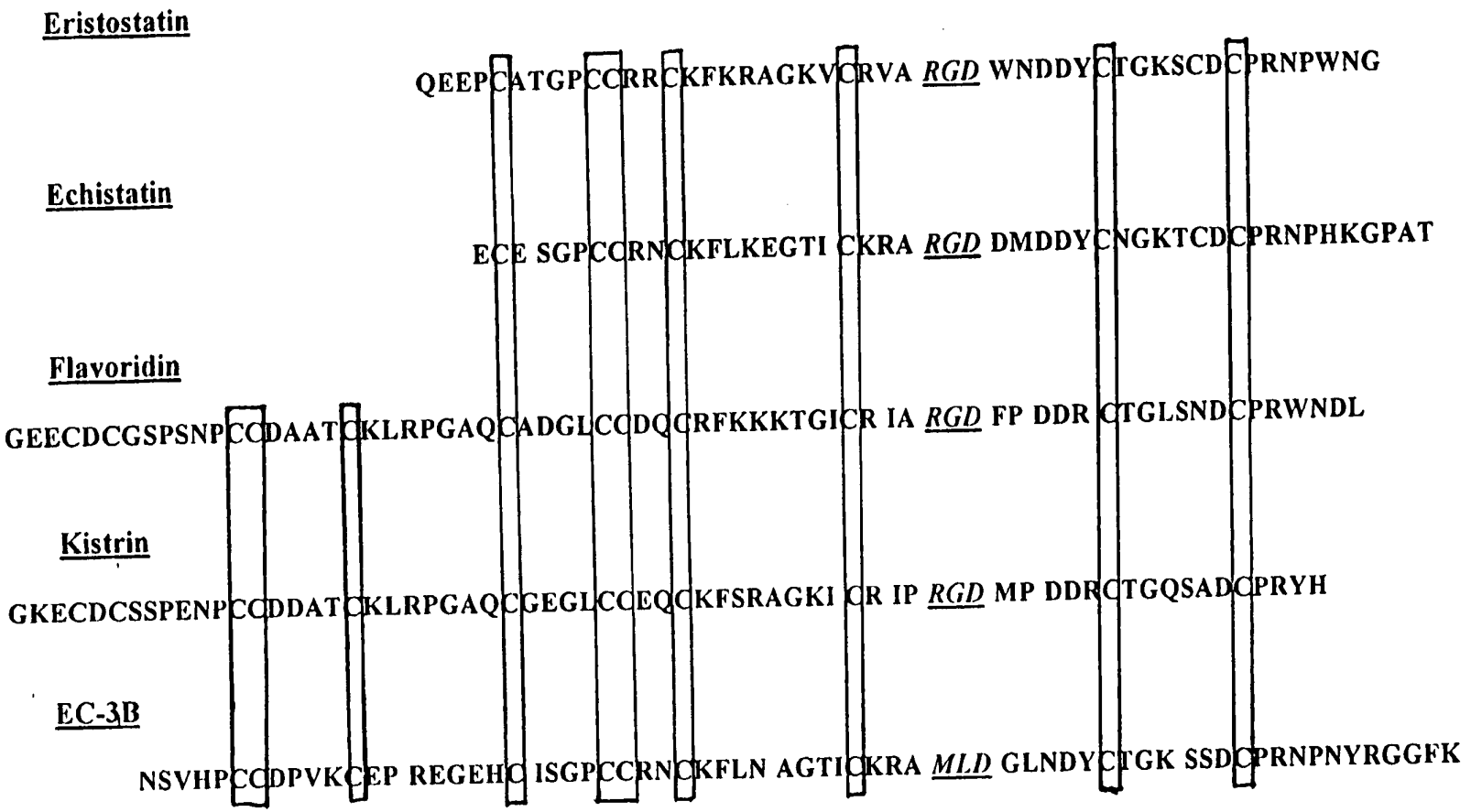
Eristostatin
QEEPCATGPCCRRCKFKRAGKVCRVA RGD WNDDYCTGKSCDCPRNPWNG
Echistatin
ECE SGPCCRNCKFLKEGTI CKRA RGD DMDDYCNGKTCDCPRNPHKGPAT
Flavoridin
GEECDCGSPSNPCCDAATCKLRPGAQCADGLCCDQCRFKKKTGICR IA RGD FP DDR CTGLSNDCPRWNDL
Kistrin
GKECDCSSPENPCCDDATCKLRPGAQCGEGLCCEQCKFSRAGKI CR IP RGD MP DDRCTGQSADCPRYH
EC-3B
NSVHPCCDPVKCEP REGEHC ISGPCCRNCKFLN AGTICKRA MLD GLNDYCTGK SSDCPRNPNYRGGFK

Fig. 3

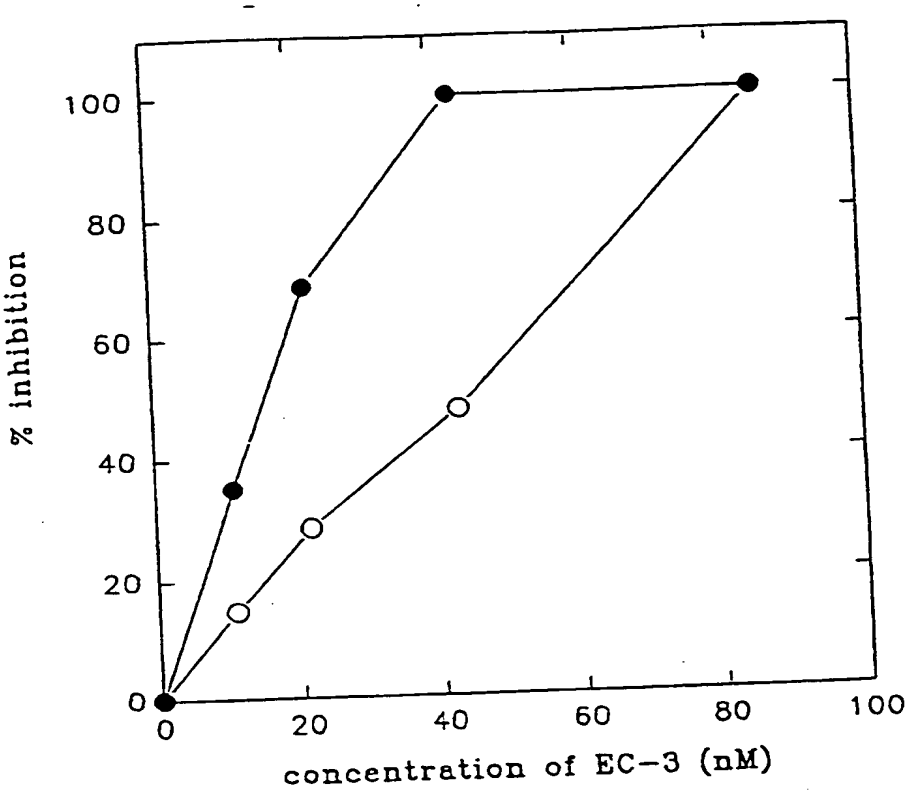
% inhibition
100
80
60
40
20
0
0
20
40
60
80
100
concentration of EC-3 (nM)

Fig. 4

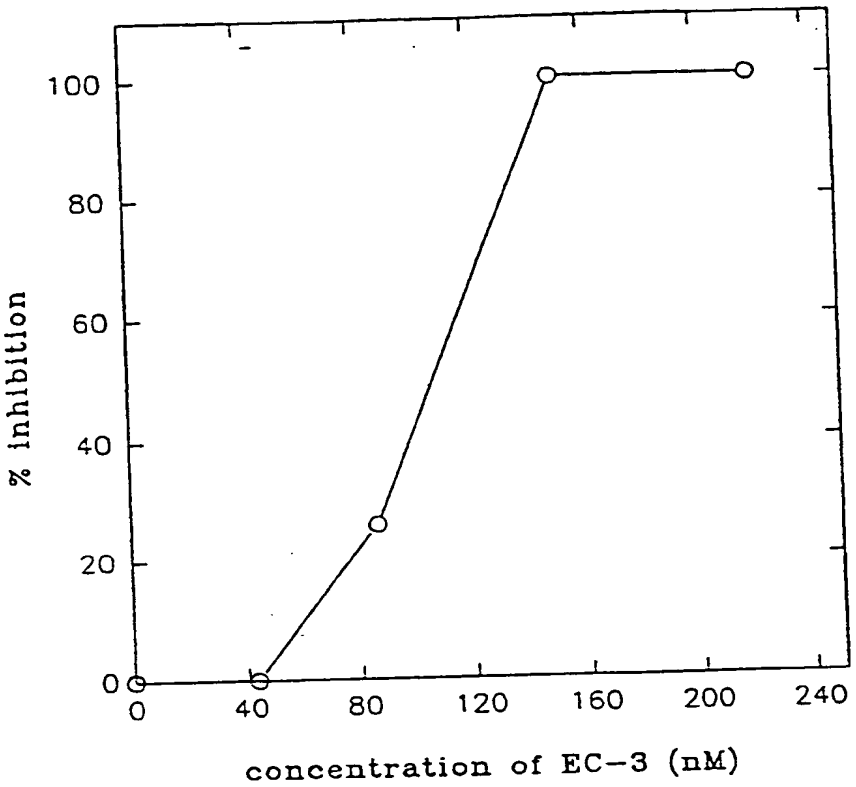
100
80
60
40
20
0
% inhibition
0
40
80
120
160
200
240
concentration of EC-3 (nM)

Fig. 5

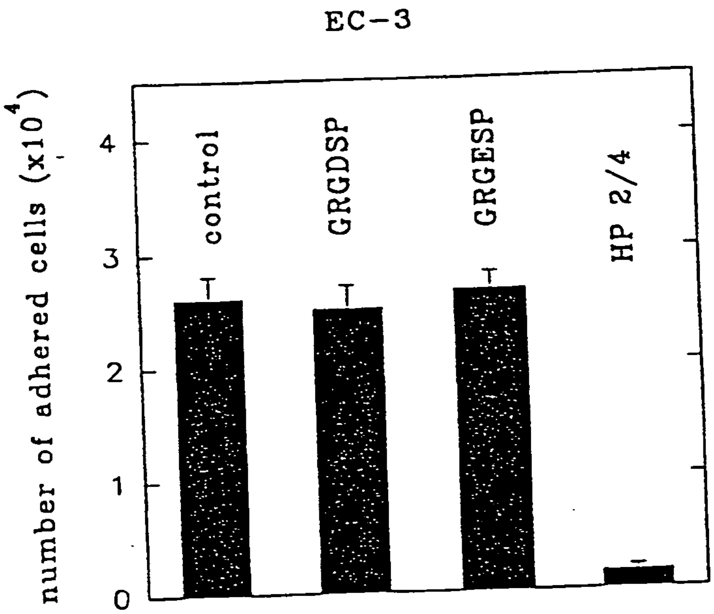

Fig. 6

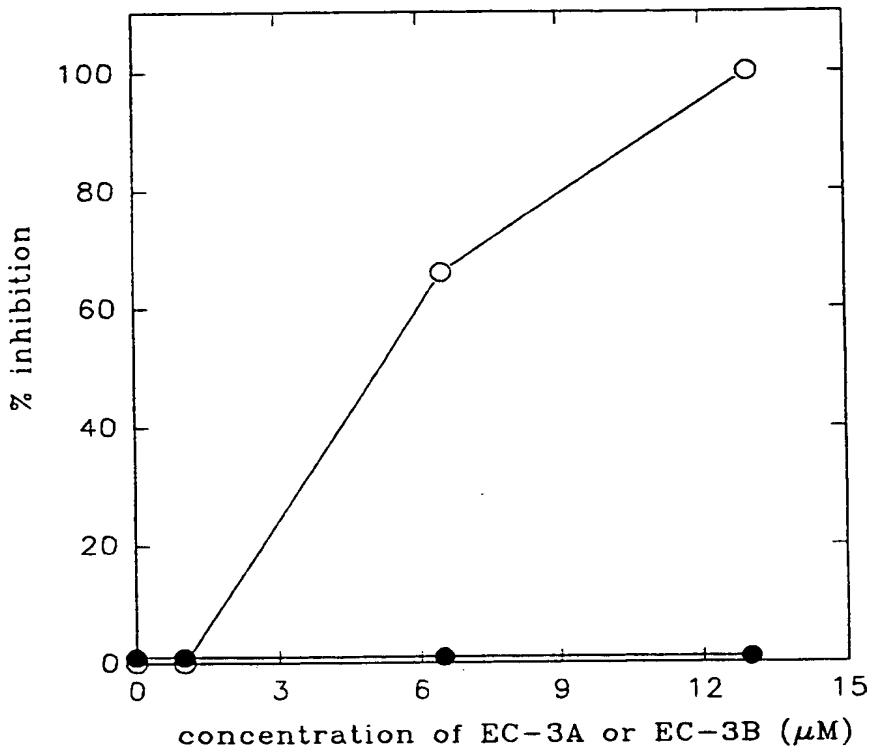
100
80
60
40
20
0
% inhibition
0
3
6
9
12
15
concentration of EC-3A or EC-3B (μM)

Fig. 7

Attorney's Docket No. 6056-236 PX1

Applicant or Patentee: Stefan Niewiarowski and Czary Marcinkiewicz
Serial r Patent No.: Not Yet Assigned
Filed or Issued: Herewith
For: EC-3 AN INHIBITOR OF $\alpha 4\beta 1$ AND $\alpha 4\beta 7$ INTEGRINS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION Temple University - Of The Commonwealth System of Higher Education

ADDRESS OF ORGANIZATION Philadelphia, PA 19122

TYPE OF ORGANIZATION

[X] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OR STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE Commonwealth of Pennsylvania)
(CITATION OF STATUTE_____)
[ ] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3) IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE_____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United State Code with regard to the above-identified invention by inventor(s) identified above

[x] the Specification filed herewith.
[ ] Application Serial No. _____, filed _____.
[ ] International Application No. _____ filed _____.
[ ] Patent No._____, issued_____.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any

concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME_____
ADDRESS_____
[ ] INDIVIDUAL [ ] SMALL BUSINESS [ ] NON-PROFIT ORGANIZATION

FULL NAME_____
ADDRESS_____
[ ] INDIVIDUAL [ ] SMALL BUSINESS [ ] NON-PROFIT ORGANIZATION

FULL NAME_____
ADDRESS_____
[ ] INDIVIDUAL [ ] SMALL BUSINESS [ ] NON-PROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

LAWRENCE C. CONNOLLY
ASSOCIATE VICE PRESIDENT
AND ASSISTANT TREASURER

NAME OF PERSON SIGNING Martin S. Dorph
TITLE IN ORGANIZATION Vice President, Chief Financial Officer and Treasurer
ADDRESS OF PERSON SIGNING Philadelphia, PA 19122

SIGNATURE_____ DATE Aug 12, 1997